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Regulation of the Trunk–Tail Patterning in the Ascidian Embryo: A Possible Interaction of Cascades between Lithium/β-Catenin and Localized Maternal Factor pem

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Embryonic cell specification and pattern formation in the ascidian embryo are controlled by prelocalized egg cytoplasmic determinants. In previous studies, we showed that overexpression of a maternal gene, *posterior end mark* (*pem*), whose transcript localizes to posterior–vegetal cytoplasm of the fertilized egg, causes a loss of the anterior and dorsal structures of the larva (Yoshida *et al.*, *Development* 122, 2005–2012, 1996). In the present study, first we observed that lithium treatment resulted in reduction of the larval tail. Lineage tracing analyses revealed that descendants of the A4.1 blastomere of the 8-cell-stage embryo (which forms the greater part of notochord and nerve cord) were missing from the tail region, that they were translocated anteriorly into the trunk region, and that the fate of the A4.1-line notochord cells had changed to endoderm. These results suggest that lithium treatment affects the trunk–tail patterning during embryogenesis by changing the cell fate of specific cell lineages. Second, we showed that lithium treatment could rescue the anterior and dorsal structures in *pem*-overexpressed larvae. This result suggests that *pem* plays a role in the patterning of the ascidian embryo via a signaling cascade that is affected by lithium. Third, we isolated an ascidian β-catenin gene and found that overexpression of β-catenin in the A4.1 blastomere had effects very similar to lithium treatment, such as reduction of the tail and anterior translocation of A4.1 descendants. These results suggest that the target of lithium is, at least in part, the Wnt-signaling cascade and that *pem* may also function via this cascade. © 1998 Academic Press

Key Words: **ascidian; lithium; trunk–tail patterning; *pem*; localized maternal factor; β-catenin.**

INTRODUCTION

Maternal information confined to a particular region of the egg cytoplasm plays a central role in the establishment of the body plan in many organisms (reviewed by Davidson, 1986; Gurdon, 1992). We have been studying the molecular nature and mode of action of such maternal information, taking advantage of the ascidian embryo (reviewed by Satoh, 1994). In ascidian eggs, fertilization evokes a dynamic rearrangement of the egg cytoplasm called ooplasmic segregation, which gives rise to the anteroposterior and dorsoventral polarity of the embryo. During this process, factors responsible for the specification of cell fate or for morphogenesis become localized to specific regions within the egg. Recent studies have provided convincing evidence of determinants responsible for the differentiation of muscle (Nishida, 1992; Marikawa *et al.*, 1994), epidermis (Nishida, 1994a), and endoderm (Nishida, 1993), factors for the establishment of the embryonic anteroposterior axis (Nishida, 1994b), and for the initiation of gastrulation (Jeffery, 1990; Nishida, 1996). In addition, recently it has been shown that inductive interactions between blastomeres play important roles in specification of certain cell types including sensory pigment cells (Nishida, 1989) and notochord (Nakatani and Nishida, 1994). Together with the unequal distribution of the determinants, such cell–cell interactions play an important role in accomplishing highly invariant development of the ascidian embryo (reviewed by Satoh, 1994).

In previous studies, using an experimental system consisting of egg fragmentation of *Ciona savignyi* (Marikawa *et*

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al., 1994, 1995), we isolated a maternal gene, *posterior end mark* (*pem*), which encodes a protein of 374 amino acids with no significant similarity to known protein (Yoshida *et al.,* 1996). The transcript is initially concentrated in the posterior–vegetal cytoplasm of the fertilized egg, and later the distribution of the transcript marks the posterior end of developing ascidian embryos. We showed that overexpression of *pem* mRNA caused a loss of the anterior and dorsal structures: i.e., the anteriormost adhesive organ and brain vesicle with sensory pigment cells (Yoshida *et al.,* 1996). The anterior neuronal cells were revealed to be translocated posteriorly and mixed with cells of the posterior portion of the CNS (Yoshida *et al.,* 1996, 1997), suggesting that anterior neuronal cells acquired posterior characteristics by receiving ectopic *pem* activity. It is possible that *pem* is required for the determination of posterior characters, and thus plays a role in the patterning of the ascidian embryo. However, the molecular mechanisms involved in embryonic patterning and the possible cascade of *pem* function remain to be elucidated.

To address these questions, we have investigated the effects of lithium on ascidian development. In amphibians, lithium is known to cause an expansion of dorsoanterior structures, leading to duplication of the axial structures or, in extreme cases, entirely dorsalized embryos with exaggerated anterior structures (Kao *et al.,* 1986). This phenotype led us to postulate that the lithium had an effect opposite to that of the overexpression of *pem* mRNA. In addition, it is known that lithium has effects on the development of diverse organisms. In sea urchins, lithium causes vegetalization of whole embryos (reviewed by Lallier, 1964) and causes isolated animal caps to form vegetal structures (Von Ubisch, 1929) and express vegetal markers (Livingston and Wilt, 1989). In addition, it was shown recently that the effect of lithium is caused by the inhibition of glycogen synthase kinase-3β (GSK3β: Klein and Melton, 1996; Stambolic *et al.,* 1996), which acts as an inhibitor of wingless/Wnt signaling cascade, although an endogenous target molecule of lithium action other than GSK3β has been reported (Berridge *et al.,* 1989; Busa and Gimlich, 1989).

In the present study, we first examined whether lithium affects the pattern formation in ascidian embryos. We observed that lithium affected the trunk–tail patterning, by affecting specifically on distinct lineages. The next question was whether there was any relationship between *pem* action and lithium treatment. We revealed that *pem*-overexpression phenotype was rescued by lithium treatment. Therefore, we tried to identify the endogenous target of lithium in the ascidian embryo. We isolated a cDNA for an ascidian β-catenin, the molecule that acts in downstream of Wnt-signaling cascade (reviewed by Moon *et al.,* 1997), as a candidate molecule of the endogenous target of lithium, and then we examined whether overexpression of this molecule can mimic the effects of lithium in the ascidian embryo. Our results suggest that the Wnt-signaling cascade plays a role in the trunk–tail patterning in ascidian embryogenesis, and that the target of lithium action is, at least in part, Wnt signaling cascade.

MATERIALS AND METHODS

Ascidian Eggs and Embryos

Ciona savignyi adults were maintained under constant light to induce oocyte maturation. Eggs and sperm were obtained surgically from the gonoduct. After insemination, eggs were reared at 18°C in Millipore-filtered seawater (MFSW) containing 50 μg/ml streptomycin sulfate. The first cleavage occurred about 1 h after insemination, and tadpole larvae hatched at about 18 h of development.

Lithium Treatment and Cleavage Arrest

Embryos were incubated in solutions of 20–40 mM LiCl-containing MFSW during the described periods of embryogenesis. To arrest the cleavage, embryos were incubated in solutions of 4 μg/ml cytochalasin B dissolved in MFSW with or without LiCl.

Isolation and Characterization of cDNA Clone for an Ascidian β-Catenin Gene

We screened *C. savignyi* red fragment cDNA library (Yoshida *et al.,* 1996) using *Xenopus* β-catenin cDNA as a probe (a gift from Dr. B. M. Gumbiner). Hybridization was performed under low-stringency conditions (hybridization: 30% formamide, 5× SSPE, 5× Denhardt's solution, 0.1% SDS, 100 μg/ml salmon sperm DNA at 37°C; wash: 6× SSC, 0.1% SDS at 37°C). The sequences of both strands of the clone were determined by the dideoxy chain termination method with Sequenase ver. 2.0 (USB; United States Biochemical Corp., Cleveland, OH). Whole-mount *in situ* hybridization was performed using digoxigenin-labeled antisense probes as described previously (Yasuo and Satoh, 1994). Control embryos hybridized with a sense probe did not show signals above background.

Injection of Synthetic Capped mRNA

pem cDNA and full-length β-catenin cDNA were subcloned into the *Eco*RI site of pBluescript-RN3 vector as described previously (Lemaire *et al.,* 1995; Yoshida *et al.,* 1996). It has been shown that the stability of β-catenin is regulated by GSK3β. The putative phosphorylation sites by GSK3β are located at the N terminus of β-catenin protein (Yost *et al.,* 1996; see Fig. 6). It has been shown that β-catenin without the GSK3β kination domain is more stable and behaves as an active form (Yost *et al.,* 1996). To construct activated β-catenin cDNA, we designed primers just downstream of the GSK3β phosphorylation sites, with an artificial initiation codon at the 5′ end. Using these primers, cDNAs without the GSK3β phosphorylation sites were amplified by polymerase chain reaction, then subcloned into pBluescript-RN3 vector. The subcloned plasmids were linearized by *Sfi*I digestion, and RNAs were transcribed by T3 RNA polymerase. To obtain a high proportion of capped mRNA, the concentration of GTP in the reaction was lowered by 10-fold, and the cap analog 7mGpppG was added at a final concentration of 0.5 mM. After DNase I digestion of the template DNA, the mRNA was purified by lithium precipitation. The precipitated RNA was rinsed three times and dissolved with DEPC-treated H_2O with a marker dye (1 mg/ml Fast Green).

The synthesized mRNA was injected into fertilized eggs as described previously (Marikawa *et al.*, 1995), using a micromanipulator (Model MP-1, Narishige Sci. Instr. Lab., Tokyo, Japan). The volume injected was estimated to be about 1/100 of the volume of an egg by measuring the quantity of coinjected marker dye. After injection, the eggs were allowed to develop, and the effects of the mRNA microinjection were examined.

Injection of Lineage Tracer

Normal and experimental eggs treated with LiCl were allowed to develop until the 8-cell stage; then either the right or left blastomere of the embryo was injected with 15 mg/ml solution of lysinated fluorescein–dextran (Molecular Probes, Eugene, OR). After injection, the embryos were allowed to develop into tadpole larvae, and the distribution of the lineage tracer was examined.

Immunohistochemistry and Histology

Differentiation of muscle cells in the experimental embryos was immunohistochemically monitored by a monoclonal antibody, Mu-2, which specifically recognizes myosin heavy chain (MHC) of muscle cells of ascidian embryos (Nishikata *et al.*, 1987). Embryos were fixed in cold methanol (−20°C) and then ethanol (−20°C) for 10 min each time. Immunofluorescence staining of whole-mount specimens and observation by fluorescent microscopy were carried out as described previously (Mita-Miyazawa *et al.*, 1987). In this study, fluorescein–isothiocyanate (FITC)-conjugated antibodies against mouse IgG, produced in sheep (Cappel, Durham, NC), were used as the second antibody. Differentiation of muscle cells was also monitored by histochemical staining for acethylcholine esterase (Karnovsky and Roots, 1964). Differentiation of endoderm cells was monitored by histochemical staining for alkaline phosphatase (AP; Whittaker and Meedel, 1989). Larvae and embryos were fixed in cold 70% ethanol for 30 s and stained for AP activity with 5-bromo-4-chloro-3-indolyl phosphate as the substrate.

Some of the stained specimens were embedded in polyester wax (BHD Chem., Poole, England) and sectioned for observation.

RESULTS

Lithium Affects the Trunk–Tail Patterning of Ascidian Embryos

To elucidate the molecular mechanisms involved in the pattern formation of ascidian embryos, we first examined whether lithium affects ascidian embryogenesis. Fertilized eggs were exposed to MFSW containing LiCl at various concentrations until the time of hatching. We used 20 to 40 mM LiCl, since concentrations higher than this frequently caused disturbances in the timing and pattern of cleavage in early embryos. When the embryos were treated with adequate concentrations, we did not detect any delay of the cell cycle or alteration of the cleavage pattern during early embryogenesis. Gastrulation took place normally, and the overall development was the same as that of the control embryos until the neurula stage. The effects of LiCl treatment became evident by the tailbud stage. As seen in Fig. 1, the tail became severely reduced in the lithium-treated embryos. The anterior trunk structures including the dorsal brain and pigment cells looked normal or rather enlarged (Fig. 1B). Reduction of the tail became remarkable when embryos were cultured at higher concentrations (Figs. 1C and 1D). The sensitivity to the concentration of LiCl varied depending on the batches of eggs used. In some batches of embryos, tail reduction was evident when the embryos were cultured in 20 mM LiCl solution; in other batches, cultured in 20 mM LiCl solution, the embryos were indistinguishable from untreated controls, and the tail reduction became evident when embryos were treated with higher than 40 mM LiCl. These embryos hatched normally, and differentiation of epidermal cells was evident because they were enclosed with larval tunic material (Figs. 1B–1D). Development of the adhesive organ was also evident in lithium-treated larvae. However, sometimes reduction in the formation of the adhesive organ was also seen when embryos were treated with high concentrations of LiCl solution (Fig. 1D). This may be due to the increase of the endoderm cell population, as will be discussed in the next section. Differentiation of muscle cells was revealed by the immunohistochemical detection of myosin-heavy chain (Fig. 1E) and the histochemical detection of acethylcholine esterase (Fig. 1F). Differentiation of endoderm cells was revealed by the histochemical detection of alkaline phosphatase (Fig. 1G).

For the determination of whether these effects were specific to lithium, embryos were treated in MFSW containing either 20 or 40 mM KCl, $MgCl_2$ or $CaCl_2$. We did not observe any of the phenotypes seen in the LiCl-treated embryos (data not shown). These results suggest that the effects found in the LiCl-treated embryos were specifically caused by lithium. In addition, to identify the sensitive period required for the tail reduction, we treated the embryos with LiCl during various periods of development. The tail reduction was seen when the embryos were treated with LiCl between the 8-cell and 32-cell stages (data not shown). This suggests that the sensitive period for the tail reduction is between the 8-cell and 32-cell stages.

These results suggest that lithium can act in some patterning mechanisms in ascidian embryos.

Reduction of the Tail by LiCl Is Caused by Anterior Translocation of A4.1 Derivatives from the Tail Region

As described above, lithium treatment caused severe reduction of the tail in ascidian embryos. It is possible that this phenotype was caused by the alteration of cell fates or by a change in the pattern formation of the posterior, tail-forming region. The ascidian tail contains notochord, nerve cord, endodermal strand, and three rows of striated muscle cells (Satoh, 1994). These structures are derived from the A4.1 and B4.1 blastomeres of the 8-cell-stage embryo. Notochord and nerve cord are formed mainly from A4.1, and muscle and endodermal strand are formed mainly from B4.1 (Nishida and Satoh, 1983; Nishida, 1987). To determine what happened to the cells that constitute the tail structure, we carried out lineage tracing analyses of these blastomeres in lithium-treated embryos.

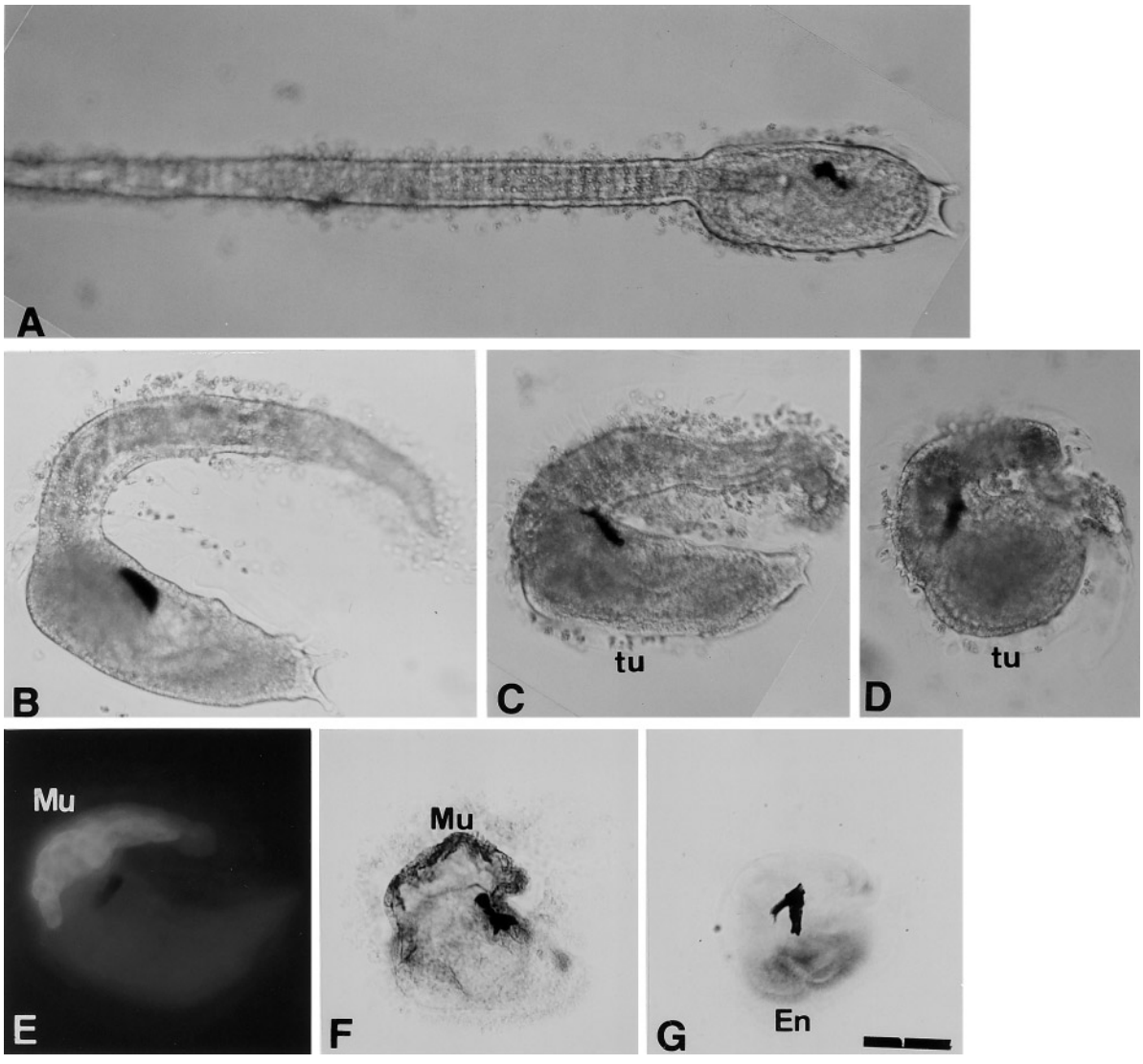

FIG. 1. Effects of lithium treatment on ascidian embryogenesis. (A) A control larva cultured in MFSW. (B) A larva cultured in MFSW containing 20 mM LiCl. (C, D) Larvae cultured in MFSW containing 40 mM LiCl. The tail is severely reduced in the lithium-treated embryos. Reduction of the tail became remarkable when embryos were treated with higher LiCl concentrations. Larval tunic (tu), a marker for the differentiation of epidermal cells, was seen around the larvae. Sensory pigment cells developed in the trunk region. Development of the adhesive organ is also evident; however, sometimes a reduction in formation of the adhesive organ was also seen when embryos were treated with high concentrations of LiCl (D). (E) Staining with Mu-2 antibody showing the expression of myosin-heavy chain, a marker for muscle cell (Mu) differentiation, in a lithium-treated larva. (F) Histochemical detection of acetylcholine esterase, another marker for the muscle cell (Mu) differentiation, in a lithium-treated larva. (G) Differentiation of endoderm cells (En) demonstrated by histochemical detection of alkaline phosphatase. Scale bar, 100 μm.

We injected the lineage tracer into A4.1 and B4.1 blastomeres of normal as well as lithium-treated embryos, and followed the cell fate of the blastomeres. The results showed that the cell fate of the A4.1 blastomeres in the lithium-treated embryos was different from that of the controls. In the control embryos, A4.1 gave rise to endoderm in the trunk and to notochord, nerve cord, and two muscle cells in the tail region (Fig. 2A). In the lithium-treated embryo, descendants of A4.1 blastomeres of the tail region were reduced (Fig. 2B) or missing (Figs. 2C and 2D) from the tail region. In these embryos, A4.1 progenitors were seen only in the trunk region, where the A4.1 blastomere usually contributes to endoderm cells.

In contrast, the cell fate of the B4.1 blastomere was not altered by lithium treatment; descendants of the B4.1 blastomere, which contributes mainly to tail muscle cells

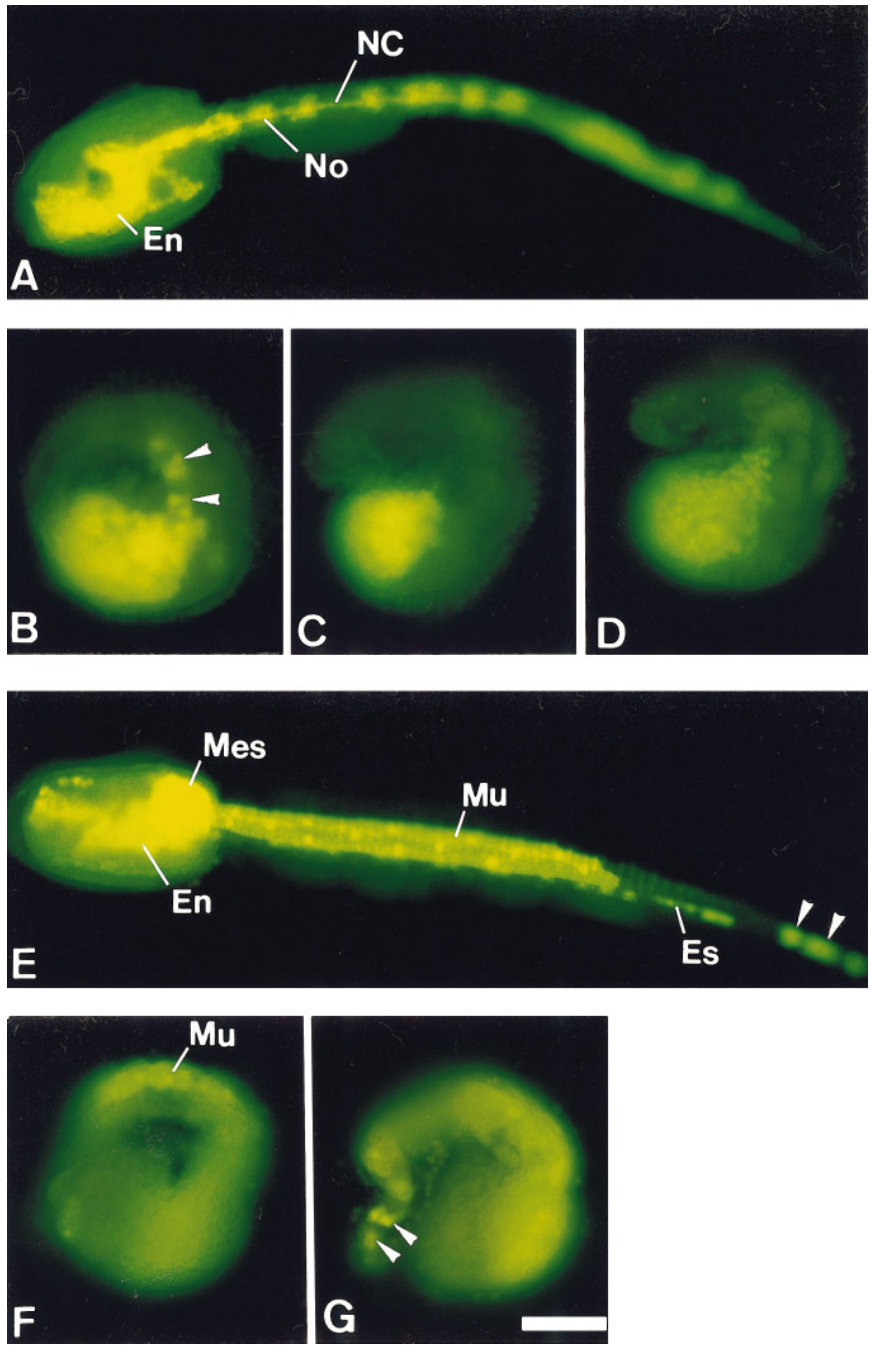

FIG. 2. Lineage tracing analyses of lithium-treated embryos. (A) A control larva showing that A4.1 progenitor cells form trunk endoderm (En), tail nerve cord (NC), notochord (No), and two muscle cells. (B–D) Distribution of A4.1 progenitor cells in lithium-treated larvae. (B) Few A4.1-derived notochord cells are seen in the tail region (arrowheads). (C, D) No A4.1 progenitors are seen in the tail region. (E) A control larva showing that B4.1 progenitor cells form trunk endoderm (En), mesenchyme (Mes), tail muscle (Mu), endodermal strand (Es), and four notochord cells (arrowheads). (F, G) Distribution of B4.1 progenitor cells in lithium-treated larvae. (F) B4.1-derived muscle cells (Mu) are seen in the tail region. (G) B4.1-derived notochord cells are seen at the tip of the tail (arrowheads). Scale bar, 100 μm.

(Fig. 2E), were found in the tail region in lithium-treated embryos (Fig. 2F). This is consistent with the finding that differentiation of muscle cells occurred normally in the lithium-treated embryos (Figs. 1E and 1F). In addition, the B4.1 blastomere (either right or left) forms four notochord cells at the tip of the tail (Fig. 2E). These B-line notochord

cells were seen at their normal sites in the lithium-treated embryos (Fig. 2G). The B4.1 blastomere also gives rise to endodermal strand in the tail (Fig. 2E). We could not confirm whether these cells were normal or affected in the lithium-treated embryos because of the large quantity of muscle cells.

These observations revealed that lithium specifically affected the cell fate of A4.1 descendants in the tail; that is, in the lithium-treated embryos, A4.1-derived cells (mainly notochord and nerve cord cells) were missing from the tail region, and these cells were translocated anteriorly into the trunk region. Because descendants of the B4.1 blastomeres were not affected by lithium treatment, lithium affects the pattern formation of the embryo by action specific to descendants of A4.1 lineage. The defect of tail structures seems to be due mainly to loss of notochord, since the notochord is a supporting organ in the tail.

Lineage tracing analyses revealed that descendants of the A4.1 blastomere were seen only in the trunk region in the lithium-treated embryos, where A4.1 mainly contributes to endoderm cells in normal embryos. However, we did not observe any notochord-like cells in the trunk region, although notochord cells are distinguishable from endoderm or other cell types by their morphology (Nakatani and Nishida, 1994). It is possible that the fate of notochord cells was changed to endoderm cells. To explore this possibility, we examined sections of lithium-treated embryos. The trunk region of the lithium-treated embryos was organized in a manner similar to that of the controls (Figs. 3A and 3B), although all of the A4.1-derived cells were located in the trunk. There were no populations of cells with notochord features. This suggests that anteriorly translocated notochord precursors differentiated to endoderm cells.

In addition, we examined the fate of notochord precursors at the 110-cell stage by taking advantage of cleavage-arrested embryos. In the 110-cell stage, notochord precursors are located anterior to the endoderm precursors (Fig. 3C). It is known that even if cleavage is arrested at this stage, the development proceeds and each blastomere differentiates autonomously to express tissue-specific markers when they reach the appropriate stage of development (Crowther and Whittaker, 1983). The pattern of expression of tissue-specific markers in the arrested embryos follows precisely the pattern of cell lineage at the stage when their cleavage was arrested. Thus, it is possible to examine the fate of a blastomere at a specific stage of development by arresting the cleavage. Normal and lithium-treated embryos were arrested at the 110-cell stage and allowed to develop until control larvae hatched, and then endoderm cell differentiation in these embryos was examined by the histochemical detection of alkaline phosphatase. In normal embryos, the domain which expressed alkaline phosphatase reflected the domain of endoderm precursors at the 110-cell stage (Fig. 3D). In the lithium-treated embryos, the domain with alkaline phosphatase activity was extended anteriorly into the notochord-forming region compared to normal embryos (Figs. 3E and 3F). This strongly suggested that the fate of notochord cells changed to endoderm cells, which eventually expressed alkaline phosphatase. Together with the results of lineage analyses, these findings indicate that it is highly likely that lithium caused translocation of A4.1-derived notochord cells anteriorly to the trunk region, and changed their fate to endoderm cells.

We therefore concluded that the effects of lithium treatment in the ascidian embryo are as follows: (1) reduction of the tail, (2) anterior translocation of the descendants of the A4.1 blastomere of the tail, and (3) change of cell fate of the A4.1-derived notochord to endoderm cells in the trunk.

Lithium Treatment Can Rescue Anterior and Dorsal Structures in pem-Overexpressed Embryos

As discussed above, lithium caused anterior translocation of A4.1-derived notochord and nerve cord cells. In previous studies, we showed that overexpression of *pem* mRNA causes loss of the anterior and dorsal structures: i.e., the anteriormost adhesive organ and brain vesicle with sensory pigment cells, while *pem* overexpression does not affect the development of the tail structure. It has been shown that loss of the anterior–dorsal structures is due to posterior translocation of those cells into the tail region. We postulate that the effect of lithium on development is, at least partially, opposite to the *pem*-overexpression phenotype. We therefore analyzed the relationship between these two treatments.

We microinjected 125 pg of synthetic *pem* mRNA into fertilized eggs and exposed them to MFSW containing LiCl and cultured them until the hatching stage, and then examined the formation of the adhesive organ and sensory pigment cells. The dose of injected mRNA was determined so that about 90% of the injected embryos failed to develop the adhesive organ and sensory pigment cells (Yoshida *et al.*, 1996). As was discussed above, the sensitivity to the concentration of LiCl varied depending on the batches of eggs used. Moreover, the effect of microinjection of *pem* mRNA also varied among the batches. To overcome these problems, batches of uninjected and injected eggs were treated in 0, 20, 30, or 40 mM LiCl in a series of experiments, and the effect was examined with each batch.

Figure 4 shows a result of one example of this type of experiment using a single batch of eggs. In a group cultured in normal MFSW (i.e., *pem*-injected), only 1 of the 11 embryos developed the adhesive organ, and 2 developed sensory pigment cells (Fig. 4A). In a group cultured in MFSW containing 20 mM LiCl, 4 of the 12 embryos developed the adhesive organ, and 9 embryos developed sensory pigment cells (Fig. 4B). In a group cultured in MFSW containing 30 mM LiCl, 9 of the 14 embryos developed the adhesive organ, and 11 developed sensory pigment cells (Fig. 4C). In a group cultured in MFSW containing 40 mM LiCl, 6 of the 10 embryos developed the adhesive organ, and all of the embryos developed sensory pigment cells (Fig. 4D). With higher concentrations of LiCl, tail reduction became evident in treated larvae, but the formation of anterior–dorsal structures was evident (Figs. 4D and 4G). These results indicate that the anterior and dorsal struc-

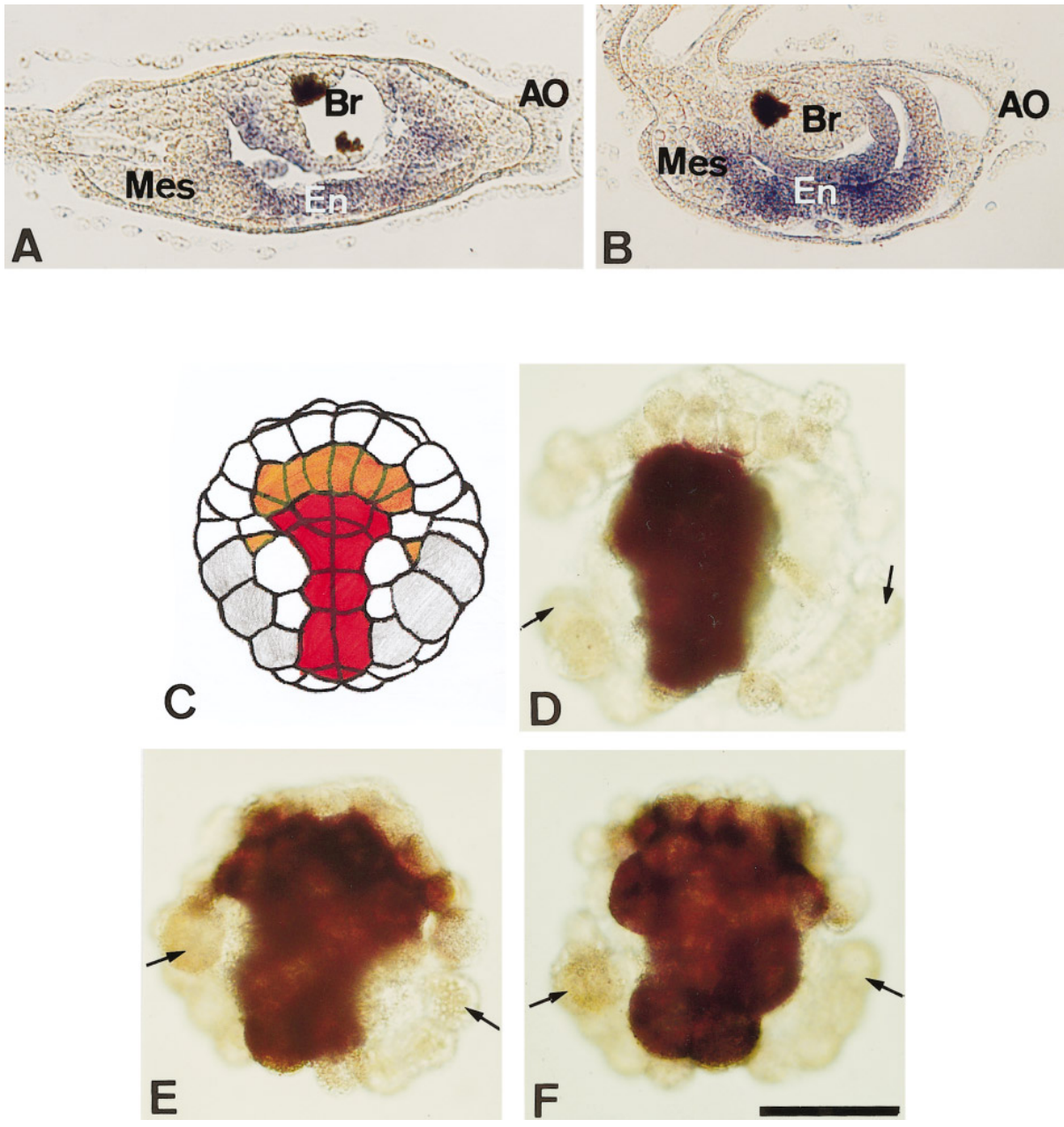

FIG. 3. The fate of anteriorly translocated notochord cells in lithium-treated embryos. (A) Section of the trunk region of a control larva. Endoderm cells (En) were stained by histochemistry for alkaline phosphatase. (B) Section of the trunk region of a lithium-treated larva. The trunk region is organized in a manner similar to that of the controls, and there are no populations of cells with notochord features. AO, adhesive organ; Br, brain; En, endoderm; Mes, mesenchyme. (C) Diagram illustrating the positions of notochord precursors (shown in orange) and endoderm precursors (shown in red) at the 110-cell stage. Notochord precursors are located anteriorly in relation to the endoderm precursors. Muscle precursors are shown in gray. (D–F) Distribution of endoderm precursors of the cleavage-arrested embryo that was cultured in normal or LiCl-containing MFSW. The shape of the arrested embryo reflected the 110-cell-stage embryo. In addition, muscle precursors (arrows), the large blastomeres which contained dark-colored myoplasm, were used as the landmark of posterior side of the arrested embryo. (D) Control embryo of which cleavage was arrested at the 110-cell stage and allowed to develop in normal MFSW until unoperated larvae hatched. Endoderm cell differentiation was detected by histochemistry for alkaline phosphatase. The domain which expresses alkaline phosphatase reflects the domain of endoderm precursors at this stage. (E, F) Embryos that were arrested at the 110-cell stage and allowed to develop in MFSW containing 20 mM LiCl. In these embryos, the domain with alkaline phosphatase activity was extended anteriorly into the region that forms notochord in the normal embryo. Scale bar, 100 μm.

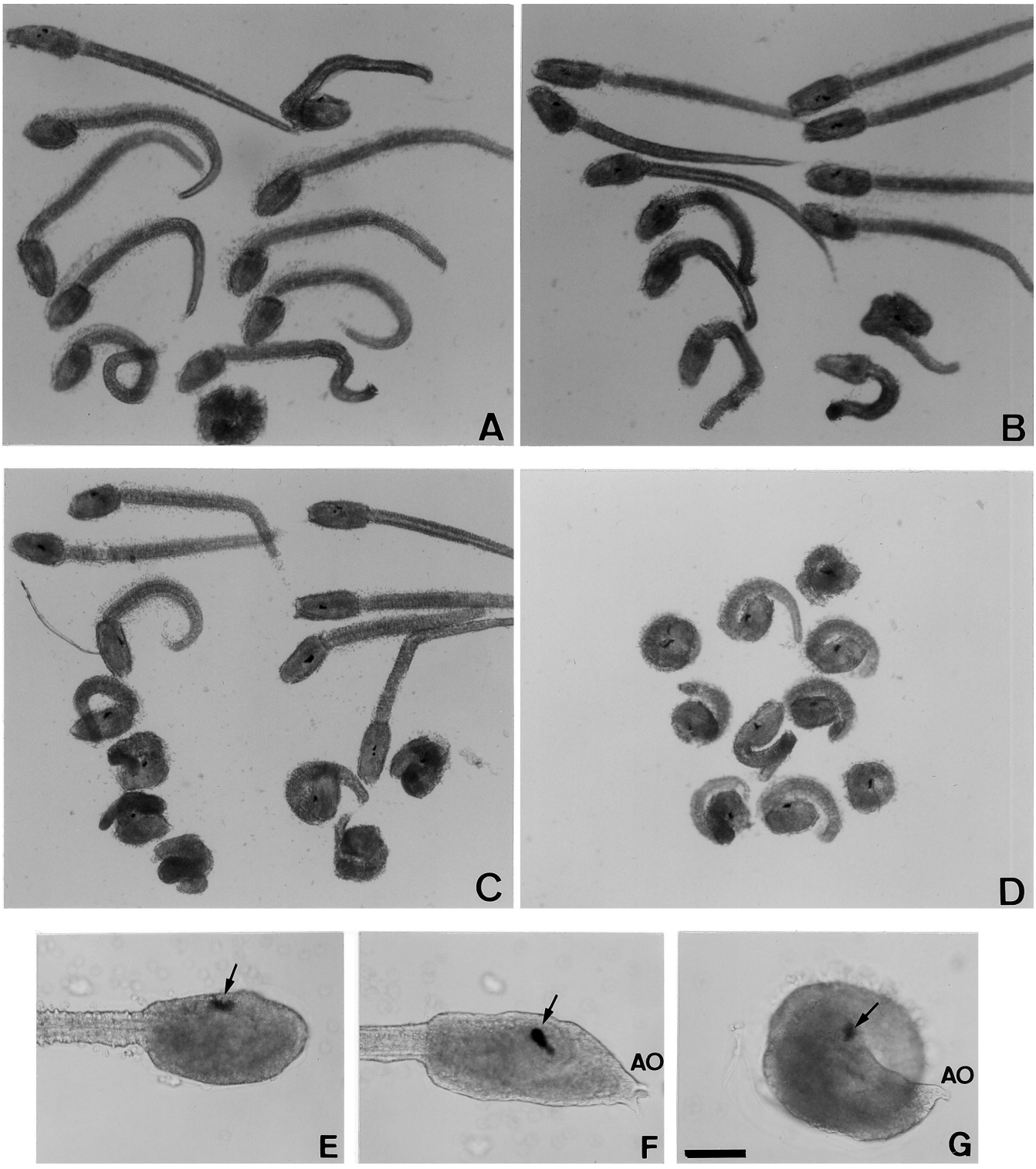

FIG. 4. Rescue of anterior–dorsal structures in *pem*-overexpressed larvae by lithium treatment. Eggs were injected with *pem* mRNA and cultured in MFSW containing various concentrations of LiCl. (A) A group cultured in normal MFSW. (B) A group cultured in MFSW containing 20 mM LiCl. (C) A group cultured in MFSW containing 30 mM LiCl. (D) A group cultured in MFSW containing 40 mM LiCl. See text for details. (E, F) Trunk regions of rescued larva. Larva that retained (E) pigment cells (arrow), or (F) pigment cells (arrow) and the adhesive organ (AO). (G) With a higher concentration of LiCl, tail reduction occurs but development of pigment cells (arrow) and that of the adhesive organ (AO) are evident. Scale bar, 100 μm.

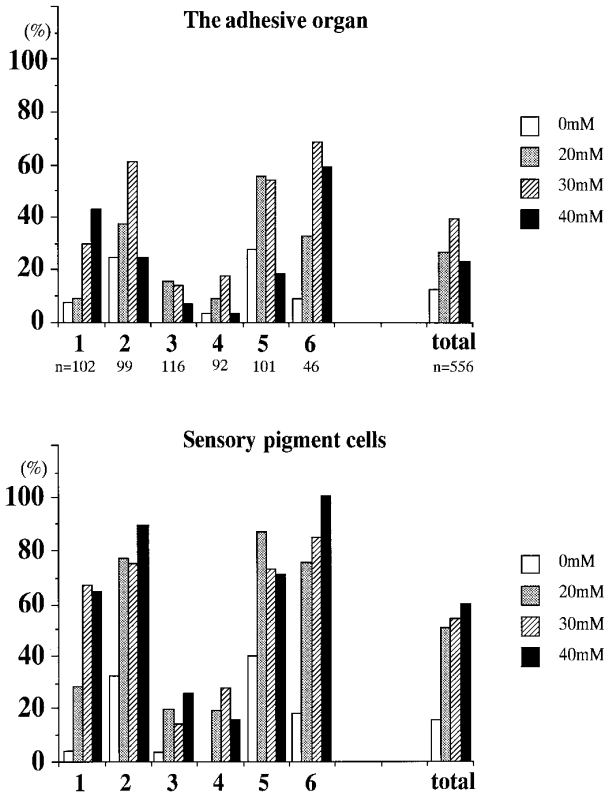

FIG. 5. The percentage of embryos that retained the adhesive organ and sensory pigment cells in *pem*-overexpressed embryos treated in MFSW with various concentration of LiCl. Results from six batches are shown. These results indicate that lithium treatment rescued the anterior–dorsal structures in a posterior-to-anterior direction in *pem*-overexpressed embryos. The right column shows the total of all of the experiments without considering the batch differences. See the text for details.

tures were formed in the *pem*-overexpressed larvae when they were treated with lithium during embryogenesis. As a result, morphologically normal trunk structure was rescued in *pem*-overexpressed embryos (Fig. 4F). Therefore, treatment with lithium significantly antagonized the *pem* overexpression.

On the other hand, the effect of lithium was not antagonized by *pem* overexpression. That is, tail reduction occurs with the same frequency in uninjected and *pem*-RNA-injected larvae when treated with the same concentration of LiCl (data not shown). This result suggests that lithium affects molecules which act downstream of *pem* in the embryogenesis.

It is noteworthy that we observed larvae with pigment cells (Fig. 4E) or with pigment cells and the adhesive organ (Fig. 4F). We did not observe any larvae with the adhesive organ but without pigment cells. This suggests that structures were recovered from the posterior side.

Figure 5 shows a summary of the experiments with six batches, with the percentage of embryos that retained the anterior and dorsal structures. In general, the percentage of embryos that retained the adhesive organ and pigment cells increased with higher concentrations of LiCl in MFSW. There were differences in the sensitivity to lithium treatment among the batches. For example, in batches 2 and 5, there was not much difference in the percentage of pigment cell formation between 20 and 40 mM treatments. Therefore, 20 mM treatment seems to be enough to retain pigment cells in these batches. In batch 1, 20 mM treatment resulted in the rescue of 25% of the embryos, and 30 mM treatment resulted in the rescue of 65%, suggesting that the eggs of batch 1 were less sensitive to lithium treatment than those of batches 2 and 5. In batches 3 and 4, the development itself was abnormal in many embryos, resulting in a low rate of rescue. The right column in Fig. 5 shows the total data of all of these experiments without considering the batch differences, indicating that treatment with lithium rescued the anterior–dorsal structures in *pem*-overexpressed embryos. In addition, in all of the experiments, the adhesive organ was recovered at a concentration higher than that at which pigment cells were recovered. This is consistent with the fact that we observed no embryos with the adhesive organ but without pigment cells, suggesting that these structures were recovered from the posterior side.

The percentage of embryos in which the adhesive organ was rescued first increased, but sometimes decreased with higher concentrations of LiCl. Reduction in the formation of the adhesive organ was also noted when uninjected embryos were treated with higher concentrations of LiCl solution (Fig. 1D). It is possible that this defect of the adhesive organ is the result of a morphological change of the trunk, possibly due to the increase of trunk endoderm cells by a change of notochord cell fate to endoderm.

These results suggest that *pem* plays a role in the establishment of the anteroposterior pattern in the ascidian embryo via a signaling cascade that lithium affects.

Overexpression of β-Catenin in A4.1 Cells Causes Anterior Translocation of Their Progenitor Cells

Our results indicate that lithium and *pem* affect some molecular mechanisms responsible for pattern formation in ascidian embryos. Therefore, it is important to understand the endogenous target of lithium in ascidian embryos. It has recently shown that lithium inhibits glycogen synthase kinase-3β (GSK3β: Klein and Melton, 1996; Stambolic *et al.*, 1996). GSK3β is a component of wingless/Wnt-signaling cascade and acts as an inhibitor of this pathway by destabilizing β-catenin, the downstream component of the Wnt-signaling cascade (reviewed by Moon *et al.*, 1997). It was shown that factors active in wingless/Wnt-signaling cascade cause dorsalization of *Xenopus* embryos. Inhibition of GSK3β (Dominguez *et al.*, 1995; He *et al.*, 1995; Pierce and Kimelman, 1995) and overexpression of β-catenin, the downstream target of GSK3β, each causes the dorsalization of *Xenopus* embryos (Funayama *et al.*, 1995). In addition, underexpression of β-catenin by antisense oligonucleotides

causes ventralization, showing that β-catenin activity is necessary for the dorsal axis formation in *Xenopus* embryos (Heasman *et al.*, 1994). These results indicate that lithium causes dorsalization of *Xenopus* embryos by inhibiting GSK3β, thus activating β-catenin. It is possible that lithium affects ascidian embryogenesis under similar mechanisms. Therefore, we isolated an ascidian homolog of β-catenin and examined whether, as in amphibians, overexpression of this molecule can mimic the lithium effects in ascidian embryos. We screened a cDNA library of *C. savignyi* egg fragments (Yoshida *et al.*, 1996) with low stringency using *Xenopus* β-catenin cDNA as a probe (a gift from Dr. B. M. Gumbiner). As shown in Fig. 6, nucleotide and deduced amino acid sequences revealed that the cDNA obtained was of an ascidian β-catenin. Northern analysis against gonad poly(A)$^+$ RNA identified a single transcript of about 2.7 kb (data not shown). Therefore it is highly likely that the cloned 2.7-kb cDNA encodes the full-length β-catenin protein. Whole-mount *in situ* hybridization revealed that the transcript distributed uniformly throughout the embryogenesis (data not shown).

We tested the effects of overexpression of β-catenin by microinjecting its synthetic mRNA. As mentioned above, the stability of β-catenin is regulated by GSK3β. The putative phosphorylation sites by GSK3β are located at the N terminus of β-catenin protein, and the domain with the phosphorylation sites was highly conserved in *C. savignyi* β-catenin (Fig. 6). It has been shown that β-catenin without the N-terminal region of the GSK3β phosphorylation sites is more stable and behaves as an active form (Yost *et al.*, 1996). We therefore produced β-catenin cDNA deprived of the N-end region (and thus without the phosphorylation sites of GSK3β, then the protein is thought to act as the constitutive active form). The mRNA for the full-length (full) and deleted (delN) cDNAs was then synthesized *in vitro* and injected.

Neither full nor delN β-catenin mRNA showed any specific effect on embryogenesis when microinjected into fertilized eggs. We then injected RNAs into each blastomere of the 8-cell-stage embryo. It was revealed that microinjection of delN β-catenin mRNA into either A4.1 (left) or A4.1 (right) blastomere caused reduction of the tail (Figs. 7A and 7B). The grade of the reduction varied somewhat among the individual larvae. Their morphology looked very similar to that of the lithium-treated embryos. The reduction of the tail became more pronounced when delN β-catenin mRNA was injected into both the A4.1 and A4.1 blastomeres (Fig. 7C). Figure 8 shows the grade of the tail reduction produced by injection of delN β-catenin mRNA, measured by the ratio of head and tail length (tail length/head length). When delN β-catenin mRNA was injected into either the A4.1 or the A4.1 blastomere, 66 of 90 embryo (76.6%) showed reduction of the tail. When the mRNA was injected into both the A4.1 and A4.1 blastomeres, 100% of the embryos showed tail reduction of a more pronounced grade. Injection of full β-catenin mRNA or control β-galactosidase (lacZ) mRNA did not cause such effects (Fig. 8). In addition, injection of delN mRNA into the B4.1 blastomere did not cause any specific effects on embryogenesis (data not shown).

We then examined the cell lineage of β-catenin-overexpressing A4.1 blastomere by coinjecting delN β-catenin mRNA and lineage tracer into either the A4.1 or the A4.1 blastomere. We observed that notochord and nerve cord, which are derived from the A4.1 blastomere, were reduced (Fig. 7D) or missing (Figs. 7E and 7F) from the tail region. In these embryos, descendants of the A4.1 blastomere were seen only in the trunk region, where the A4.1 blastomere contributes to endoderm cells. As was also seen in lithium-treated embryos, these A4.1 progenitors looked like endoderm, and we found no notochord-like cells among the A4.1 progenitors. Table 1 shows the summary of this analysis. In 81.8% of the injected larvae, A4.1 progenitors were seen exclusively in the trunk. In 12.7% of the larvae, few A4.1 progenitors were seen in the tail, and in 5.5% of the larvae, the A4.1 cell fate was indistinguishable from that of the wild-type. These results suggest that A4.1-derived cells in the tail region translocated anteriorly by receiving ectopic β-catenin activity. This effect was the same as that seen in the lithium-treated embryos (see Fig. 2). It is therefore likely that lithium affected patterning of the embryo via ectopic activation of β-catenin. These results suggest that the Wnt-signaling cascade plays a crucial role in the trunk-tail patterning of the ascidian embryo, and that the endogenous target of lithium described above is, at least in part, Wnt signaling cascade.

DISCUSSION

In this study, we addressed molecular mechanisms involved in the pattern formation of the ascidian embryo. First, we showed that the lithium treatment of early embryos resulted in reduction of the tail. Lineage tracing analyses revealed that derivatives of the A4.1 blastomere of the 8-cell-stage embryo, which forms notochord, nerve cord, and two muscle cells at the tip of the tail, were missing from the tail region and translocated anteriorly to the trunk. The analysis using cleavage-arrested embryos suggested that the fate of the notochord cells was changed to endoderm cells of the anterior trunk region. These results suggest that lithium affects specific aspects of pattern formation in the ascidian embryo, as reported in many organisms such as amphibians and sea urchins. This system will serve as a model for study of the mechanisms responsible for pattern formation in the ascidian embryo.

Second, we observed a reciprocal relationship between overexpression of *pem* mRNA and lithium treatment in formation of the anterior and dorsal structures of the ascidian embryo. We found that lithium treatment could rescue the anterior and dorsal structures in *pem*-overexpressed embryos. This result suggests that *pem* plays

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   1 GGGATCCTGTCGTATTATCCGTTAACTTTGAATAGTTGTGAACATCCACAAGGTGTCAGTATGGCTGACTTGATGATGAATCGAAATGAG  90
                                                                M  A  D  L  M  M  N  R  N  E   10
  91 ATGGTGCCTGACGCGAAAGCCCAAGTTCGAATGTGGAACTGGGATTCTGGGATCCAATCTGGAGCAACCACCGCCACCCCGTCTATAAGT 180
     M  V  P  D  A  K  A  Q  V  R  M  W  N  W  D  S  G  I  Q  S  G  A  T  T  A  T  P  S  I  S   40
 181 GGGGCTAGTCACCATGATGGGAATTACCCTGATACGAACCAAGTGTTGGGCGACTGGCAGCGAGAATTTACAATGGAGGAAGTTGACCAA 270
     G  A  S  H  H  D  G  N  Y  P  D  T  N  Q  V  L  G  D  W  Q  R  E  F  T  M  E  E  V  D  Q   70
 271 ATCCACGACCAGTTTGTCCGAACTCGTGCGGACCGTGTGCGCGATGTTCTCTTCCCCGAGACAATGGACGACGGAACCCAGATCCCCAGC 360
     I  H  D  Q  F  V  R  T  R  A  D  R  V  R  D  V  L  F  P  E  T  M  D  D  G  T  Q  I  P  S   100
 361 ACGCAGTTCGACGACGGCACCACAACCTCTGTGCAACGACTCGCTGAGCCTTCGCAGCAGCTCAAGAAAGCCGTTGTCAATCTCATTAAT 450
     T  Q  F  D  D  G  T  T  T  S  V  Q  R  L  A  E  P  S  Q  Q  L  K  K  A  V  V  N  L  I  N   130
 451 TATCAGGACGACGCAGACTTGGCCACGAAAGCGATACCCGAGCTGACGGGGCTGCTTAACGATGACGACCCAGTTGTGGTGCAGCAAGCC 540
     Y  Q  D  D  A  D  L  A  T  K  A  I  P  E  L  T  G  L  L  N  D  D  D  P  V  V  V  Q  Q  A   160
 541 GCACAGATGGTCCATATGTTGTCCAAGAAAGTGGCGAGTCGACAAGCGATCAGGAATTCTCCCGCGATGGTGTCGGCCCTCGTACGCGCT 630
     A  Q  M  V  H  M  L  S  K  K  V  A  S  R  Q  A  I  R  N  S  P  A  M  V  S  A  L  V  R  A   190
 631 ATGCAGAATGCGACGGACCCAGACACCCAGCGATACTGCACCGGAGCTCTACACAACCTCTCCCACCACAAGCAAGGATTGCTATCCATC 720
     M  Q  N  A  T  D  P  D  T  Q  R  Y  C  T  G  A  L  H  N  L  S  H  H  K  Q  G  L  L  S  I   220
 721 TTCAAGTCTGGGGGAATCCCCGCACTAGTGAAGATGCTTGGATCCCCCATCGACTCGGTGATGTTCTACGCGATCACCACCCTCCACAAC 810
     F  K  S  G  G  I  P  A  L  V  K  M  L  G  S  P  I  D  S  V  M  F  Y  A  I  T  T  L  H  N   250
 811 CTCCTGCTACATCAGGAAGGAGCAAAGGAGGCGGTCAGGTTAGCCGGAGGACTCCAGAAGATGGTCTTCCTCCTCTGCAGGGACAACGTG 900
     L  L  L  H  Q  E  G  A  K  E  A  V  R  L  A  G  G  L  Q  K  M  V  F  L  L  C  R  D  N  V   280
 901 AAGTTCCTCGCTATCGATACCGATTGTCTGCAGATCCTCGCGTACGGGAACCAGGAAAGCAAGTTGATTATCCTGGCGAGCAACGGGCCG 990
     K  F  L  A  I  D  T  D  C  L  Q  I  L  A  Y  G  N  Q  E  S  K  L  I  I  L  A  S  N  G  P   310
 991 CAGGAGTTGGTACGAATCATGCGAACGTACGATTATGAGAAGCTGCTGTGGACGACCAGTCGTGTCATCAAGGTCTTGTCGGTTTGCTCC 1080
     Q  E  L  V  R  I  M  R  T  Y  D  Y  E  K  L  L  W  T  T  S  R  V  I  K  V  L  S  V  C  S   340
1081 AACAACAAGCCTGCCATTGTGGAAGCTGGCGGCATGCAAGCACTGGGTCTCCACCTGGGCTCCCGCTCGCAGAGGTTGCTCCAGAACTGC 1170
     N  N  K  P  A  I  V  E  A  G  G  M  Q  A  L  G  L  H  L  G  S  R  S  Q  R  L  L  Q  N  C   370
1171 TTGTGGAGTTTGCGGAACTTGTCGGACGCTGGTACGAAGCAGGACCAAGTGGAGAATCTACTGCAGATGCTGGTGCAGCTTTTATCATCG 1260
     L  W  S  L  R  N  L  S  D  A  G  T  K  Q  D  Q  V  E  N  L  L  Q  M  L  V  Q  L  L  S  S   400
1261 AACGACATCAACGTGGTGACGTGTGCGGCAGGCATCCTCAGTAATCTCACCTGCAACAACATGAGCAACAAGACCAGGGTTTGCCAGGTT 1350
     N  D  I  N  V  V  T  C  A  A  G  I  L  S  N  L  T  C  N  N  M  S  N  K  T  R  V  C  Q  V   430
1351 GGAGGCATCGAGGCACTTGTACGCACGGTGTTGCAAGCTGGGGATCGGGAAGACATCACCGAACCCTCTGTGTGCGCGTTGCGTCACCTC 1440
     G  G  I  E  A  L  V  R  T  V  L  Q  A  G  D  R  E  D  I  T  E  P  S  V  C  A  L  R  H  L   460
1441 ACCTCCCGTCACCCCGACGCCGAGATGGCCCAGAACGCCGTGCGACTCCACTACGGGCTCCCTGTGTTGGTCAAGTTGCTTCACCCACCA 1530
     T  S  R  H  P  D  A  E  M  A  Q  N  A  V  R  L  H  Y  G  L  P  V  L  V  K  L  L  H  P  P   490
1531 TCCCGATGGCCCCTCATCAAGGCAGTCGTTGGGCTCATCCGCAACCTTGCGCTATGCTCGGCCAACCACGGGGCTCTGCGTGAGCACGGG 1620
     S  R  W  P  L  I  K  A  V  V  G  L  I  R  N  L  A  L  C  S  A  N  H  G  A  L  R  E  H  G   520
1621 GCGATCCCGCGCTTGGTCCAGCTGCTCATGCGCGCCCACCAAGACACCCAGCGTCGTACGAGCCTCGCGTCCAGTCACAGCCATATGTCG 1710
     A  I  P  R  L  V  Q  L  L  M  R  A  H  Q  D  T  Q  R  R  T  S  L  A  S  S  H  S  H  M  S   550
1711 GCTCACGTGGCGCACTCGGCGCATGTGGACGGGGTGCGCATGGAGGAGATCGTGGAGGGGACGACCGGCACGCTGCACATCATGGCCCGG 1800
     A  H  V  A  H  S  A  H  V  D  G  V  R  M  E  E  I  V  E  G  T  T  G  T  L  H  I  M  A  R   580
1801 GAAGCCCACAGCAGAGCCGTCATCCGAGGTCTCAACACGATTCCGCTCTTCGTGCAACTGCTGTACTCACAGGTTGAAAACATCCAGCGG 1890
     E  A  H  S  R  A  V  I  R  G  L  N  T  I  P  L  F  V  Q  L  L  Y  S  Q  V  E  N  I  Q  R   610
1891 GTGGCAGCAGGCGTGCTGTGTGAGTTGGCGCAGGACAAGGACAGCGCGGAGTTGATCGAAGTGGAAGGAGCAAGCACCCCACTCACCGAG 1980
     V  A  A  G  V  L  C  E  L  A  Q  D  K  D  S  A  E  L  I  E  V  E  G  A  S  T  P  L  T  E   640
1981 CTGTTGCACTCCAAGAACGAGGGGGTAGCCACGTACGCAGCTGCTGCATTGTTCCGGATGTCCGAGGACAAATCCCAGGATTACAAGAAG 2070
     L  L  H  S  K  N  E  G  V  A  T  Y  A  A  A  A  L  F  R  M  S  E  D  K  S  Q  D  Y  K  K   670
2071 AGACTTTCCGTCGAACTTACCAGCTCGCTATTCAAAGATGACGGGGCACTTTATAATGGAACGGACCTGGTCGATCACTCCATGCACGGT 2160
     R  L  S  V  E  L  T  S  S  L  F  K  D  D  G  A  L  Y  N  G  T  D  L  V  D  H  S  M  H  G   700
2161 TTCCACCACCAGATGACGCCACCGATGTCGCACCAGAGTTCCGTCAGTTCCGTCCATAATTCCCACCATTCAGGCTACCCACAAGCGGAG 2250
     F  H  H  Q  M  T  P  P  M  S  H  Q  S  S  V  S  S  V  H  N  S  H  H  S  G  Y  P  Q  A  E   730
2251 GGCCAGCAACCCATGGACTTCCAGCAGCACCTCGTGCCCCCTGCCGAGTACCCTCTCCCTGATTTGTCGAACGACTTGGACCTCGAGCCG 2340
     G  Q  Q  P  M  D  F  Q  Q  H  L  V  P  P  A  E  Y  P  L  P  D  L  S  N  D  L  D  L  E  P   760
2341 ATGATCGGAGTCCAACAACCGTGGCTTGATAGCGACCTCTAGTGTGCGAGGTAAAGCGTGGAAGAATGGATCAATCATTGAAAGCCCCCC 2430
     M  I  G  V  Q  Q  P  W  L  D  S  D  L  *                                                773
2431 AATGTACAAAATTTTATAATTGCTTTTAATACTTTGCTGAATCGAAGAAACCGTTGTTGGGTTCGAAGATTAAAATTAGATTCGAATGTT 2520
2521 TGAGATGTCTATGTGGATTTTACGGAGCTTCCGACAGTGATTATGACGTCACAGCCAGTTCGTTTTCTATTGTTTTTATGACGTAGCCTT 2610
2611 AAATCGCTTGTAACCATAGCAACCATGTAGCCCGCGTATTTCGTGTGAATAAACTCAAAATGTACAGCAAAAAAAAA              2687
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FIG. 6. Nucleotide and deduced amino acid sequence of *Ciona savignyi* β-catenin cDNA clone. The 2687-bp insert includes a single open reading frame that encodes a polypeptide of 773 amino acids. The putative protein contains potential glycogen synthase kinase 3β phosphorylation sites at the N-terminal region, which are indicated by boxes. The termination codon is shown by an asterisk. A putative polyadenylation signal is underlined.

a role in establishing the pattern of the ascidian embryo via a signaling cascade that lithium affects.

Third, we isolated an ascidian homolog of β-catenin as a candidate for endogenous target of lithium and examined the effect of overexpression of this molecule in ascidian embryogenesis. We found that overexpression of activated β-catenin in the A4.1 blastomere caused reduction of the tail. In these embryos, the A4.1-derived notochord and nerve cord were missing in the tail, suggesting that A4.1 descendants translocated anteriorly to the trunk region. These effects were very similar to those of the lithium treatment. Therefore, the target of lithium is, at least in part, the Wnt signaling cascade.

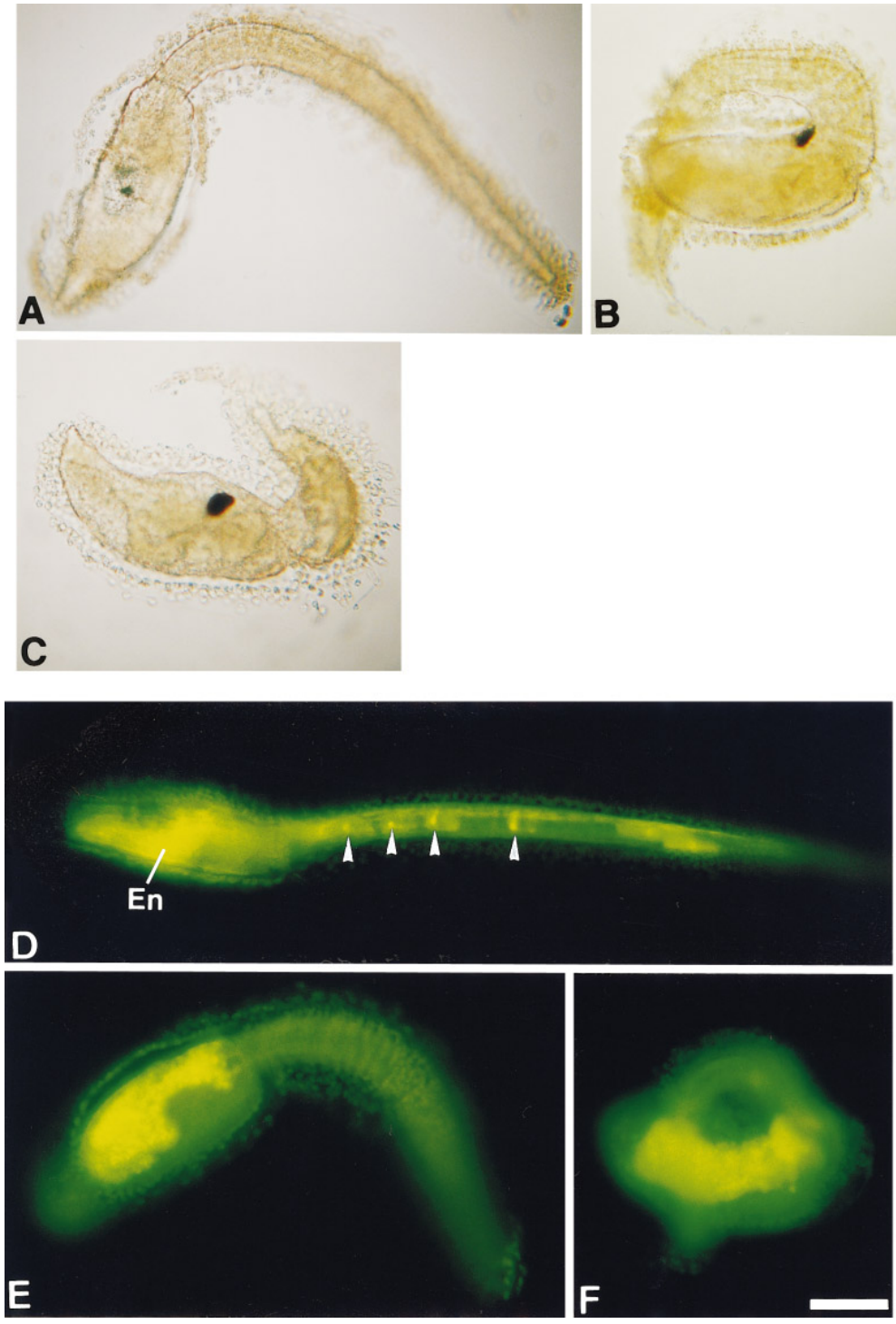

FIG. 7. Effects of β-catenin overexpression on ascidian embryogenesis. (A, B) delN β-catenin mRNA was injected into one of the A4.1 blastomere pair (note that the ascidian embryo is bilaterally symmetrical). Reduction of the tail is evident in the larvae, and the grade of reduction varied among individuals. The morphology of the larvae resembles that of lithium-treated larvae. (C) delN β-catenin mRNA was injected into both of the A4.1 blastomere pair. Tail reduction is much more conspicuous compared to when delN mRNA was injected into only one of the blastomere pair. (D–F) Change of the fate of A4.1 progenitor cells by overexpression of delN β-catenin mRNA. (D) Few A4.1-derived notochord cells are seen in the tail region (arrowheads). (E, F) No A4.1 progenitors are seen in the tail region. En, endoderm. Scale bar, 100 μm.

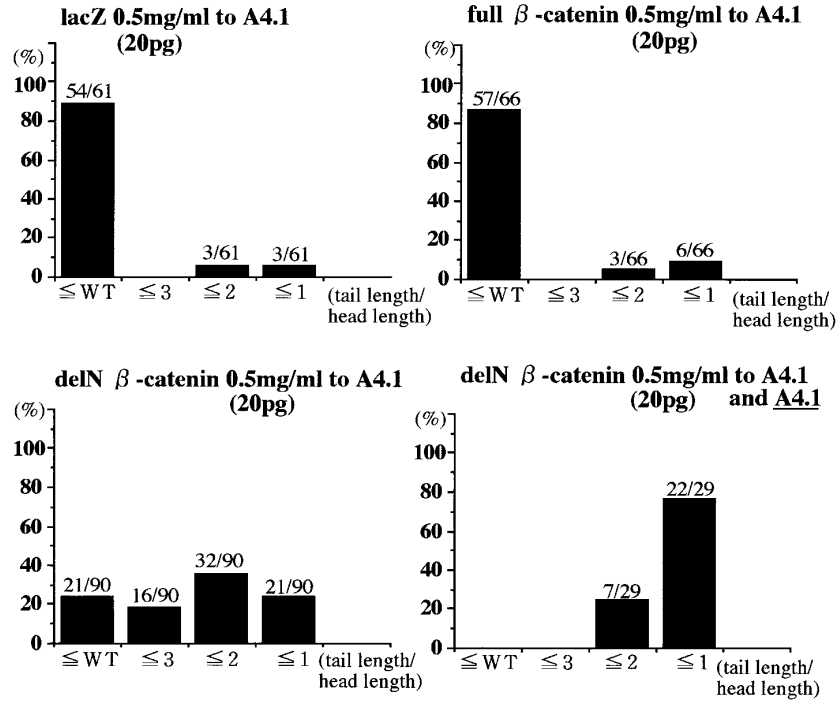

FIG. 8. Tail reduction by β-catenin overexpression, measured by the ratio of head and tail length (tail length/head length).

Mechanisms Involved in the Trunk–Tail Patterning in Ascidian Embryos

In ascidians, notochord cells are mainly derived from the A4.1 blastomere pair of the 8-cell-stage embryo (32 of the 40 cells). These A-line notochord cells are called primary lineage cells. The B4.1 blastomere pair also contributes to 8 notochord cells at the tip of the tail, which are called secondary lineage cells. Similarly, muscle cells are mainly formed from the B4.1 blastomere, and are called primary lineage. The A4.1 blastomere pair and B4.2 blastomere pair both contribute to four muscle cells at the tip of the tail, and these are called secondary lineage cells. It has been suggested that different mechanisms are involved in differentiation of notochord cells between the primary and secondary lineage (Nakatani and Nishida, 1994; Nakatani *et al.*, 1996) as well as muscle cells in the different lineages (Nishida, 1990).

We found that lithium treatment specifically affected A4.1 progenitors; that is, primary lineage notochord cells, nerve cord cells and secondary lineage muscle cells were missing from the tail, and they translocated anteriorly into the trunk. Moreover, the fate of notochord cells changed to endoderm cells. In contrast, most of the B4.1 progenitors of the tail, namely, primary lineage muscle cells and secondary lineage notochord cells, were not affected by lithium treatment. These results indicate that the specification of the cell fate of primary and secondary lineage cells is regulated differentially according to the cell origins. Therefore, it may be concluded that the patterning mechanism acts on descendants of a distinct lineage, regardless of the kind of the tissue. We also examined the relationship between overexpression of *pem* mRNA and lithium treatment, and found that lithium treatment could rescue the anterior and dorsal structures in *pem*-overexpressed em-

TABLE 1

The Cell Fate of the A4.1 Blastomere Which Was Injected delN β-Catenin mRNA

	Distribution of A4.1 progenitors		
	Normal	Trunk and a few in tail	Trunk only
delN β-catenin, 20 pg to A4.1	3/55 (5.5%)	7/55 (12.7%)	45/55 (81.8%)

Note. The percentages of the larvae in which a reduced number of A4.1-derived cells were found in the tail region (Fig. 7D) or no A4.1 progenitors were found in the tail region (Figs. 7E and 7F) were scored. See text for details.

bryos. This result suggests that *pem* and the target of lithium are components that are commonly involved in the anteroposterior patterning of the ascidian embryo. The molecules on which *pem* and the target of lithium act are an important research topic for future investigations.

Mechanisms Involved in the Specification of the Notochord and Endoderm Cell Fate

We observed that the fate of A-line notochord cells was changed to endoderm cells of the anterior trunk region by lithium treatment, and that this lithium effect could be mimicked by overexpression of β-catenin. It has been shown that endoderm cells differentiate autonomously dependent on prelocalized determinants (Nishida, 1993), and that inductive interaction which occurs at 32-cell stage is required for notochord cell differentiation (Nakatani and Nishida, 1994). Notochord precursors that do not receive an inductive signal fail to differentiate into notochord, or any other kind of tissue including endoderm (Nakatani and Nishida, 1994). Therefore, the change in notochord cell fate that is produced by lithium treatment is not caused simply by the failure of inductive interaction. It has been shown that the expression of *As-T* gene, the ascidian *Brachyury*, begins in notochord precursors at the 64-cell stage, immediately after the inductive interaction occurs (Yasuo and Satoh, 1993, 1994). When synthetic *As-T* mRNA is overexpressed in notochord precursors, these cells differentiate into the notochord without inductive interactions. In addition, when *As-T* mRNA is expressed uniformly within the embryo, the fate of endoderm and nerve cord cells is changed to notochord cells (Yasuo and Satoh, 1998). Together with our results, it is suggested that the endoderm and notochord precursors share similar characteristics, and that some common mechanisms are at work in the specification of the endoderm and the notochord in addition to the unequal segregation of determinants and inductive interactions. In addition, our results regarding the overexpression of β-catenin suggested that this mechanism is associated with the Wnt signaling cascade. It would be of interest to address the mechanism underlying the specification of the notochord and the endoderm in relation to the Wnt signaling cascade.

Possible Molecular Cascades Involved in the Patterning of Ascidian Embryos

As discussed above, it is important to elucidate the molecular target of the action of lithium because this information will clarify the patterning mechanisms in ascidian embryos. In *Xenopus*, it has been shown that lithium mimics embryonic responses to Wnts (Christian and Moon, 1993; Stambolic *et al.*, 1996). The target molecule was recently suggested to be GSK3β (Klein and Melton, 1996) which plays a role in the Wnt-signaling cascade by regulating the activity of β-catenin (reviewed by Moon *et al.*, 1997). Therefore, we isolated an ascidian β-catenin gene as a candidate molecule for the target of lithium action, and we examined whether overexpression of β-catenin can mimic the effects of lithium in ascidian embryos. We found that microinjection of activated β-catenin mRNA into one of the A4.1 blastomere pair, or both of the blastomere pair, caused reduction of the tail, similarly the case of lithium-treated embryos. We also observed that notochord and nerve cord, which are derived from the A4.1 blastomere, were reduced or missing from the tail region in these embryos. Thus, overexpression of β-catenin in the A4.1 blastomere caused effects very similar to those of the lithium treatment. These results strongly suggest that the Wnt-signaling cascade plays a role in the trunk–tail patterning of the ascidian embryo, and that the endogenous target of lithium described above is, at least in part, the Wnt signaling cascade.

We observed that lithium treatment rescued the anterior and dorsal structures in *pem*-overexpressed embryos. It is possible that overexpression of β-catenin can also rescue the *pem*-overexpression phenotype. We tried to test this possibility by microinjecting β-catenin mRNA into *pem*-overexpressed embryos. We coinjected delN mRNA with *pem* mRNA into fertilized eggs. We also injected *pem* mRNA at the 1-cell stage, and then injected delN mRNA into a4.2 or A4.1 blastomeres at the 8-cell stage. So far, however, we have not succeeded in rescuing the *pem*-overexpression phenotype by β-catenin overexpression. One possibility for this result is that the lithium target on the tail structures is different from that which rescues *pem* activity because it has been shown that lithium inhibits the signaling cascade via inositol 1,4,5-triphosphate by inhibiting inositol monophosphatase (Berridge *et al.*, 1989; Busa and Gimlich, 1989). However, we suspect that lithium treatment antagonized *pem* activity by activating the Wnt signaling cascade. This speculation is based on the following observations. Injection of delN β-catenin mRNA into the A4.1 blastomere of 8-cell stage mimicked the lithium effect on the tail structure; however, injection into fertilized eggs, or full-length β-catenin mRNA into fertilized eggs or the A4.1 blastomere, did not mimic the lithium effect. The sensitive period when lithium affects the tail structure is the period between 8-cell and 32-cell stages. This means that overexpression of β-catenin is effective only when the activated form is injected into specific blastomeres and with precise timing. Although we could not determine the sensitive period during which lithium antagonizes *pem* activity, it may be later stages of development; therefore, injection of β-catenin mRNA at the 8-cell stage could not rescue the *pem*-overexpression phenotype. The period of lithium action that antagonizes *pem* activity should be defined in further studies. It would be also important to elucidate the relationship between *pem* action and other factors involved in the Wnt-signaling cascade, such as transcription factor LEF-1 (Behrens *et al.*, 1996; Huber *et al.*, 1996) or XTcf-3 (Molenaar *et al.*, 1996), which acts with β-catenin.

Most ascidian species have a tadpole larva with a tail structure, but some ascidian species have evolved a tailless

(anural) larva (reviewed by Jeffery and Swalla, 1990; Satoh and Jeffery, 1995). Since the lithium-treated larvae also have reduced tail structure, we want to know if there is any similarity or difference between these two phenotypes. Jeffery and Swalla utilize two closely related species as an experimental system, *Molgula oculata* and *M. occulta*; the former produces tailed (urodele) larvae while the latter produces tailless (anural) larvae. The molecular mechanisms that cause the change of developmental mode from the urodele to the anural type have been investigated (for example, Swalla *et al.*, 1993; Swalla and Jeffery, 1996). *M. occulta* larvae does not develop sensory pigment cells, and the presumptive notochord and muscle cells fail to differentiate and undergo morphogenetic movements leading to tail formation (Jeffery and Swalla, 1990). Lithium treatment also affected the tail structure, but the effect was mainly on the notochord and nerve cord. Other tail features such as muscle were not affected by lithium treatment. Thus, at present we suspect that reduction of the tail structure by lithium treatment is caused by a mechanism other than that which causes the change of developmental mode to the anural type.

Our present results elucidated some molecular components involved in the pattern formation accomplished in the early stage of ascidian embryogenesis. These findings will contribute to the clarification of the patterning mechanism of ascidian development.

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